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Year: 2013

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## **Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis**

Lutterotti, A ; Yousef, S ; Sputtek, A ; Stürner, K H ; Stellmann, J P ; Breiden, P ; Reinhardt, S ; Schulze, C ; Bester, M ; Heesen, Ch ; Schippling, S ; Miller, S D ; Sospedra, M ; Martin, R

**Abstract:** Multiple sclerosis (MS) is a devastating inflammatory disease of the brain and spinal cord that is thought to result from an autoimmune attack directed against antigens in the central nervous system. The aim of this first-in-man trial was to assess the feasibility, safety, and tolerability of a tolerization regimen in MS patients that uses a single infusion of autologous peripheral blood mononuclear cells chemically coupled with seven myelin peptides (MOG1-20, MOG35-55, MBP13-32, MBP83-99, MBP111-129, MBP146-170, and PLP139-154). An open-label, single-center, dose-escalation study was performed in seven relapsing-remitting and two secondary progressive MS patients who were off-treatment for standard therapies. All patients had to show T cell reactivity against at least one of the myelin peptides used in the trial. Neurological, magnetic resonance imaging, laboratory, and immunological examinations were performed to assess the safety, tolerability, and in vivo mechanisms of action of this regimen. Administration of antigen-coupled cells was feasible, had a favorable safety profile, and was well tolerated in MS patients. Patients receiving the higher doses ( $>1 \times 10^9$ ) of peptide-coupled cells had a decrease in antigen-specific T cell responses after peptide-coupled cell therapy. In summary, this first-in-man clinical trial of autologous peptide-coupled cells in MS patients establishes the feasibility and indicates good tolerability and safety of this therapeutic approach.

DOI: <https://doi.org/10.1126/scitranslmed.3006168>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-85434>

Journal Article

Accepted Version

Originally published at:

Lutterotti, A; Yousef, S; Sputtek, A; Stürner, K H; Stellmann, J P; Breiden, P; Reinhardt, S; Schulze, C; Bester, M; Heesen, Ch; Schippling, S; Miller, S D; Sospedra, M; Martin, R (2013). Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis. *Science Translational Medicine*, 5(188):188ra75.  
DOI: <https://doi.org/10.1126/scitranslmed.3006168>

# **Induction of Antigen-specific Tolerance by Autologous Myelin Peptide-Coupled Cells – A Phase I Trial in Multiple Sclerosis Patients**

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**One Sentence Summary:** The first-in-man trial of tolerization with antigen-coupled cells for treatment of MS demonstrates the feasibility, safety and tolerability of this novel therapeutic approach and shows first evidence for an antigen-specific tolerization upon dosing with  $1 \times 10^9$  or higher numbers of antigen-coupled cells.

**Abstract:** The aim of this first-in-man trial was to assess the feasibility, safety and tolerability of a novel tolerization regimen in MS patients, that employs a single infusion of autologous peripheral blood mononuclear cells chemically coupled with seven myelin peptides (MOG1-20, MOG35-55, MBP13-32, MBP83-99, MBP111-129, MBP146-170 and PLP139-154). An open-label, single center, dose escalation study was performed in 7 relapsing-remitting and 2 secondary progressive MS patients, who were off-treatment for standard therapies. All patients had to show T cell reactivity against at least one of the myelin peptides used in the trial. Neurological, MRI, laboratory and immunological examinations were performed to assess the safety, tolerability and *in vivo* mechanisms of action of this regimen. We followed the overall patient immune response as well as responses to myelin antigens prior to and after administration of peptide-coupled PBMC. Overall, administration of antigen-coupled cells was feasible, had a favorable safety profile and was well tolerated in MS patients. Compared to the pre-treatment observation period there was no increase in clinical and MRI parameters of disease activity by this regimen. Patients receiving the higher doses ( $>1 \times 10^9$ ) of peptide-coupled cells had a decrease in antigen-specific T cell responses following peptide-coupled cell therapy. In summary, this first-in-man clinical trial of autologous peptide-coupled cells in MS patients, establishes the feasibility, and indicates good tolerability and safety of this novel therapeutic approach.

## Introduction

Approaches to induce antigen-specific tolerance in multiple sclerosis (MS) hold the promise to stop the pathogenic autoimmune response, thus preventing disease activity while at the same time avoiding the potentially severe side effects, which are associated with many of the currently employed immunotherapies (1, 2). In MS the primary target antigen/s is/are not known for certain, but it is well accepted that proteins within the myelin sheath, such as myelin basic protein (MBP), myelin oligodendrocyte protein (MOG) and proteolipid protein (PLP) are important targets of the autoreactive immune response (3). However, the target epitopes of myelin proteins differ between MS patients, and it is likely that the myelin-specific T cell reactivity may change over time (4-6). In relapsing-remitting animal models of MS, chronic demyelination leads to the generation of new T cell responses against multiple endogenous antigens, a process called epitope spreading, and these newly generated T cells are able to induce relapses, which can be inhibited by tolerance to the spread epitope (7, 8). Therefore, it is reasonable to assume, that the efficacy of antigen-specific therapies will not only depend on knowledge of the specific target antigens, but also on the ability to block epitope spreading at an early stage and thereby stop diversification of T cell autoreactivity. Consequently, antigen-specific therapies should simultaneously target previously activated autoreactive T cells and also naïve autoreactive T cells specific for multiple myelin epitopes.

Antigen-coupled cell tolerance is a tolerization strategy with a long-standing and excellent track with regard to efficacy and safety in several experimental models of autoimmune diseases, transplantation tolerance and allergic disease (9, 10). Antigen-specific tolerance is induced through carrier cells, which are pulsed with antigens in the presence of the chemical cross linker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (9, 11-15). Studies in experimental

autoimmune encephalomyelitis (EAE), an animal model of MS, have proven that a single i.v. injection of syngeneic splenocytes coupled with encephalitogenic myelin peptides/proteins is highly efficient in inducing antigen-specific tolerance *in vivo* (15-22). In EAE this protocol not only prevented animals from disease, but even effectively reduced the onset and severity of all subsequent relapses when given after disease induction (17, 18, 23-25). As a major advantage of the therapy, tolerance can be simultaneously induced to multiple epitopes using a cocktail of myelin peptides (23).

With the aim to induce antigen-specific tolerance in MS we adopted this promising tolerization strategy to treat patients with MS (supplementary Fig. S1). We have established a procedure for the manufacture of antigen-coupled cells using autologous peripheral blood mononuclear cells (PBMC) as carriers. Seven myelin peptides (MBP13-32, MBP83-99, MBP111-129, MBP146-170, MOG1-20, MOG35-55, PLP139-154), which were previously identified as important targets of autoreactive T cells in MS (26-30) were coupled to the surface of PBMC by EDC.

We assessed the feasibility, safety and tolerability of antigen-coupled cell tolerance in a first-in-man, open-label, single center clinical trial in relapsing-remitting (RR) and secondary-progressive (SP) MS patients (ETIMS-trial). Only patients, who had an antigen-specific T cell response against at least one of the peptides used in the trial, were eligible for treatment in the study and T cell reactivity was analyzed before and after treatment.

## Results

### *Preparation and infusion of antigen-coupled cells*

At the day of study drug administration,  $4-10 \times 10^9$  peripheral blood mononuclear cells (PBMC) and 200 ml autologous plasma were isolated from MS patients by leukapheresis (Cobe Spectra;

TerumoBCT, Belgium). The manufacturing process of peptide-coupled cells was started immediately, and all steps were performed under good medicinal practice (GMP) conditions in standard blood bags while maintaining a closed system (Fig. 1C). During this process PBMC were chemically coupled with 7 myelin peptides (MBP13-32, MBP83-99, MBP111-129, MBP146-170, MOG1-20, MOG35-55, PLP139-154). A detailed description of the manufacturing process can be found in the Methods. At the end of the manufacturing process, the autologous peptide-coupled cell product was re-suspended in 100 ml of autologous plasma for infusion (ETIMS cell product). Prior to infusion samples were analyzed to meet the following release criteria of the cellular product: residual EDC <1,9 µg/ml, endotoxin <0.5 EU/ml (Endosafe, Charles River, Wilmington, USA), viability (> 70% propidium iodide negative cells measured by flow cytometry), pH (7,2–7,8) and absence of aggregates (microaggregates <1/µl). The peptide-coupled cells were administered within 4 h after the last step of preparation of study drug product. The manufacturing process was feasible in all patients and all cell products fulfilled the release criteria.

***Adverse events including changes in vital signs, blood chemistry and blood cell counts after treatment with ETIMS cell product***

In order to determine safety and tolerability of treatment with ETIMS cell product, we evaluated the number and severity of AEs, significant changes in vital signs and changes in blood chemistry and blood cell counts. Twenty-four AEs, which were not considered MS-related, were reported at different times after treatment. The severity of these events as well as their putative relation with treatment are tabulated in Table 2. From these 24 AEs only 1 may be related to ETIMS product. Patient 2 reported a metallic flavor during infusion of study drug. The AE was

graded as mild, but relation to study drug could not be excluded. The same patient had an irritation of a punctured vein, which had been caused by the leukapheresis procedure but not the ETIMS product, since it was not administered through that vein. One serious adverse event (SAE) not related with ETIMS treatment, a diverticulitis of sigma, occurred in patient 1 six weeks after administration of study drug.

No significant changes in vital signs were observed after ETIMS treatment. Hematological analyses did not reveal either clinically significant abnormalities following treatment in blood chemistry (data not shown) or blood cell counts (Fig. 2). The global number of eosinophils, basophils, neutrophils, lymphocytes, monocytes and platelets was stable after treatment in all patients including the four patients receiving higher dose of antigen-coupled cells (patient 6, 7, 8 and 10).

### ***MS course after treatment with ETIMS cell product***

As an additional outcome measure for safety and tolerability we assessed if the ETIMS treatment led to worsening of MS. Worsening of MS was determined by neurological examination, i.e. occurrence of exacerbations and/or disability progression and, as the most sensitive measure, the occurrence of new T2 lesions or contrast-enhancing lesions (CELs) by magnetic resonance imaging (MRI). Since the main aim of this trial was to assess feasibility, safety and tolerability, the first six patients selected (patient 1-6) were patients with low disease activity in order to avoid that naturally occurring disease activity confounded the assessment of tolerability and safety. None of the first 6 patients treated with antigen-coupled cells (patients 1-6) showed a relapse during the first three months after treatment (Fig. 3). Concerning the three more active patients (patient 7, 8 and 10), patient 7 and patient 8 showed one MS disease exacerbation



following treatment at days 10 and 16 , respectively (Fig. 3). Both patients had high disease activity before inclusion in the study and the clinical presentation or MRI findings at the time of relapse were not uncommon with respect to prior disease history. Patient 7 presented with mild dysfunction in fine motor skills and dysaesthesia in both upper extremities. MRI revealed a CEL in the cervical spinal cord. Symptoms remitted completely following corticosteroid treatment. Patient 8 presented with dysaesthesia for temperature sensation in the right leg. MRI showed contrast enhancement in the cervical spinal cord lesion, which was already present the month before treatment. At this time the patient had a myelitis with residual paraesthesia in both legs. After corticosteroid treatment the symptom remitted. No further relapse occurred during the trial in these patients, and further clinical follow-up of the two patients with relapses disclosed reduced disease activity during the treatment period and follow-up compared to their disease activity prior to enrolment and during the baseline period.

Neurologic function remained stable in all patients during the six months following treatment. There was no increase in neurological disability measured by Expanded disability status Scale (EDSS), Scripps Neurological Rating Scale (SNRS), Multiple Sclerosis Impact Scale (MSIS29) (Figure 5) nor Multiple Sclerosis Functional Composite (MSFC).

All patients completed the MRI protocol. The number of new T2 lesions as well as CELs before and at different time points after treatment for the first 6 patients with low disease activity are summarized in Fig. 4 (upper graphs). No increase in CEL or new T2 lesions was observed in these 6 patients. Regarding the second (dose-escalation) cohort, which was composed of more active patients (patient 7, 8 and 10), new CELs as well as new T2 lesions were detected after treatment (Fig. 4). In all three patients a single new T2 lesion was detected at week 2, and, even though these were small lesions that did not look different from previously observed T2 lesions

in each patient, it cannot not be completely excluded that these were related to the ETIMS treatment. Although the number of patients is very small, the number of new T2 and CEL is lower compared to baseline in patient 8, and slightly elevated in patient 7 and patient 10. After completion of the three months protocol, all patients were followed further as part of the safety analysis. During this period one MS exacerbation was observed in patient 3 and patient 5, respectively (Fig. 3). Patient 3 presented in week 18 with a hypaesthesia in the left leg without motor deficits. A corresponding CEL was seen on the spinal cord MRI. The hypaesthesia remitted completely following corticosteroid treatment. Patient 5 presented in week 17 with mild paresis of the right hand, a symptom, which she had experienced several times previously. While it remained unclear if these symptoms constituted a relapse, they were counted as such. Clinical examination and routine laboratory ruled out acute infection and brain MRI did not show any CEL or new T2 lesion. Symptoms completely resolved after corticosteroid treatment. Finally, we want to mention that patient 6 reported a transient increase in spasticity three months after study-drug-administration, which is a common symptom in MS and induction by the treatment was unlikely at this late point.

### ***Characterization of blood cell populations after treatment with ETIMS cell product***

As a supplementary measure to determine safety and tolerability of ETIMS treatment, we also characterized by flow cytometry several relevant immune cell populations before and after treatment in whole blood obtained from the four patients receiving the higher dose of antigen-coupled cells, patients 6, 7, 8 and 10. Frequencies of monocytes, B cells, T cells, NK cells and NK-T cells before and after treatment are shown in Fig. 6A. ETIMS treatment did not induce significant changes in the percentage of any of these immune cell populations. Frequencies of

several functional CD4<sup>+</sup> T cell subsets including Th1 (IFN- $\gamma$ -producing cells), Th2 (IL-4-producing cells), Th17 (IL-17-producing cells), Tr1 (IL-10-producing cells) and regulatory T cells (Treg, FoxP3-expressing cells) were also analyzed before and after treatment. Results are summarized in Fig. 6B, and individual data of patients in the high-dose group are shown in supplemental Fig. S3. With regard to safety of the regimen it is important to note that we did not detect increases in the frequency of Th1 or Th17 CD4<sup>+</sup> T cell subsets 3 months after treatment. The frequency of Tr1, Treg and Th2 cells remained stable during the course of the study. Frequencies of several functional CD8<sup>+</sup> T cell subsets including “regulatory” CD8<sup>+</sup> T cells (CD8<sup>+</sup> CD57<sup>+</sup> ILT2<sup>+</sup> T cells) and “pro-inflammatory” CD8<sup>+</sup> T cells (CD8<sup>+</sup> CD161<sup>high</sup> T cells) were stable following ETIMS treatment (Figure 6C; individual data of patients in the high-dose group are shown in supplemental Fig. S3).

### ***Characterization of myelin-specific T cell responses after treatment with ETIMS cell product***

To address whether antigen-coupled cells had an effect on the frequency of T cells specific for the antigens used in this study, we measured antigen-specific T cell responses before and three months after treatment. The percentage of positive wells, i.e. wells with scintillation counts (CPM) higher than the mean plus 3 standard deviations (mean+3xSTDEV) of the unstimulated wells, for each peptide before and after treatment in all patients receiving the lower dose of antigen-coupled cells (patients 1-5) and in the patients receiving the higher dose (patient 6, 7, 8 and 10), is summarized in Fig. 7.

Before treatment, patients were considered to have a positive T cell response against a specific peptide if they showed 2 or more positive wells, i.e. wells with CPM > Mean+3xSTDEV of unstimulated wells. The ETIMS treatment was considered to have an effect, when a reduction of

2 or more positive wells was observed three months after treatment. Patient 6 showed positive T cell responses before treatment (more than 2 wells with  $CPM > \text{Mean} + 3 \times \text{STDEV}$  of unstimulated wells) to all seven myelin peptides (MOG 1-20, MOG 35-55, MBP 13-32, MBP 83-99, MBP 111-129, MBP 146-170 and PLP 139-154). Three months after treatment, T cell responses to all of these peptides (MOG 1-20, MOG 35-55, MBP 13-32, MBP 83-99, MBP 111-129, MBP 146-170 and PLP 139-154) were reduced (Fig. 7). Patient 7 showed positive T cell responses against four different peptides (MOG 1-20, MOG 35-55, MBP 146-170 and PLP 139-154) before treatment, and these responses were all reduced after treatment (Fig. 7). A positive response to MBP 13-32 was measured in patient 7 in a single well, and this was also reduced after treatment. Patient 8 showed positive responses before treatment against MOG 1-20, MOG 35-55, MBP 13-32, MBP 83-99, MBP 111-129 and these responses were also reduced after treatment. In patient 8 a single well was positive against MBP 146-170, and this was reduced after treatment. Finally, patient 10 showed before treatment positive T cell responses to all seven myelin peptides (MOG 1-20, MOG 35-55, MBP 13-32, MBP 83-99, MBP 111-129, MBP 146-170 and PLP 139-154), and the response to all of these peptides were reduced after ETIMS treatment (Fig. 7).

## Discussion

In this first-in-man trial we have established the feasibility of antigen-coupled cell tolerization in MS and provide evidence for the safety and tolerability of this novel therapeutic approach. Tolerization by ETIMS involves autologous PBMC pulsed with seven myelin peptides in the presence of the coupling agent EDC. EDC catalyzes the formation of peptide bonds between free amino- and carboxyl groups, thereby producing peptide-coated cells that function as highly

tolerogenic carriers. This therapy is in many aspects novel and unique including the use of a set of peptides that covers the immunodominant epitopes of different myelin proteins. Six peptides of these three myelin proteins (MOG, MBP and PLP) were chosen because they were previously shown to be targets of the high-avidity autoimmune T cell response in MS (26). MBP 83-99 was added since it has been shown to be immunodominant in MS patients by many prior studies and has been a target of previous tolerization trials (3, 31, 32). To the best of our knowledge this is the first tolerization strategy that simultaneously targets 7 peptides from 3 myelin proteins.

Previous tolerization approaches in MS patients mainly focused on single MBP peptides or MBP protein (31-33). Recently, transdermal application of 3 peptides from MBP, MOG and PLP showed efficacy in reducing myelin-specific T cell reactivity (34). Different from all other tolerization therapies antigen-coupled cell tolerance was shown to prevent epitope spreading in animal models. Further, tolerization with antigen-coupled cells has been shown to act in part independently of the MHC in mice, and may thus potentially be applicable for both human leukocyte antigen (HLA) DR15-positive and DR15-negative MS patients (35).

To translate this regimen from mice to MS patients we have developed a manufacturing process for antigen-coupled cells, which is completely performed under GMP conditions in standard blood bags while maintaining a closed system (Fig. 1 and supplementary Fig. S1). The autologous cell product can be re-infused within the same day, which renders it a feasible approach for the outpatient care setting.

For safety reasons it was requested by the regulatory authorities that we perform a very careful dose escalation starting with  $1 \times 10^3$  cells in the first patient up to the target dose of  $3 \times 10^9$  antigen-coupled cells in the last patient. We did not encounter relevant safety concerns related to the study drug and have thus met the primary endpoint set for this first-in-man study. There was a

single SAE during the 3 months core study period, which was considered not related to the therapy by both the investigators and the independent DSMB, all other AEs were graded as mild or moderate. A critical issue in antigen-specific therapies is the risk of induction of disease by the treatment as previously observed in a vaccination trial using an altered peptide ligand (31). Therefore, we aimed to include patients, who did not have highly active disease in this first-in-man trial. Since we did not observe induction of disease in the first six patients, we expanded our dose-escalation regimen with three more patients, who had clinically active disease. In two of these patients we observed exacerbations and new T2 MRI lesions 10 and 16 days after therapy (Fig. 3 and Fig. 4). Importantly, in both cases the presentation of the exacerbation was similar or identical to symptoms that the patients had experienced during recent months prior to treatment, and hence different from what had been observed in a trial using an altered peptide ligand (31). In the latter trial, 3 out of 8 patients experienced exacerbations with clinical- and/or MRI presentation that were very different from the patients' prior history, and the massive increase of MBP peptide-specific T cells left little doubt that relapses had been treatment-induced (31).

There was no increase in disability over the course of the study in any of the patients (Fig. 5). As stated above, we cannot exclude completely that the disease activity (exacerbations or new MRI lesions), which was observed within the first couple of weeks in the small group of highly active patients, was related to the ETIMS therapy, although we would have expected that it occurred even earlier in the trial, had this been the case. Of note, no further relapse occurred during the six months follow-up period in any of the highly active patients (patients 7, 8 and 10). However, the data raise the question if the dose of peptide-coupled cells should not be escalated even further.

The two observed exacerbations in patients treated with a high-dose of antigen-coupled cells are an important safety signal. Therefore, strict safety measures, both clinical and MRI, are

warranted in future applications of antigen-coupled cells during clinical trials.

Although the primary aim of the study was safety and tolerability, another important objective was to gather information on its immunologic effects. Thus, we treated only patients in whom a T cell responses towards the myelin peptides used in the trial could be measured at baseline.

Interestingly, myelin peptide-specific T cell responses at baseline were higher in those patients with ongoing inflammatory disease activity. Patients treated in the high dose group (patients 6, -

7, 8 and 10) showed a uniform reduction in myelin-specific T cell response, although a few

positive wells were measured in patients 6, 7 and 10 following treatment (Fig. 7). Therefore,

together with the above clinical/MRI data we conclude with respect to dose-finding that

minimally a dose of  $3 \times 10^9$  peptide-pulsed cells or even more should be used, and given the good

tolerability of the regimen, further dose escalation is not expected to pose problems. Three

patients (1, 7 and 8) were HLA-DR15 positive, thus a definite conclusion on the influence of the

HLA cannot be drawn. In a mouse EAE model, it has been shown that, while antigen-coupled

cells can induce tolerance independently of the MHC, tolerance induction with peptide-coupled

allogeneic cells required the administration of higher cell numbers than syngeneic cells or

repeated injections (14, 35). Thus, beside a further dose escalation, as already mentioned, future

trials should consider to give repeated injections of autologous antigen coupled cells.

The exact mechanism of action of the tolerization regimen is not yet fully understood, but there

is evidence that several distinct mechanisms are involved. It has been shown that antigen-

specific T cells encountering their cognate antigen/MHC complexes on EDC-treated cells are

anergized as a result of failure to receive adequate CD28-mediated co-stimulation (36). However,

*in vivo*, another mechanism might be more important, which is based on the fact that EDC

efficiently induces apoptosis in treated cells. Experiments in animal models suggest that

apoptotic EDC-treated cells are phagocytosed in the spleen within a few hours by antigen presenting cells residing in this organ (immature dendritic cells or monocytes/macrophages), which leads to the production of IL-10 and expression of PD-L1 on macrophages as important factors for the induction of tolerance. Additionally, the induction of T regulatory cells plays a central role in the long-term maintenance of tolerance induced by this procedure (35, 37). In our patients we observed only a slight increase in the overall frequency of regulatory T cell subsets in peripheral blood, however, larger number of patients, who are treated with a homogeneous cell number, are needed to analyze this further.

Contingent on future studies confirming the antigen-specific therapeutic effects of the regimen, antigen-coupled cell tolerance has the potential for wide applicability in different autoimmune diseases, transplantation tolerance and allergy. The feasibility and easy applicability, without the need for long term cell culture or expansion of cells *ex vivo* are major advantages of this approach. The clinical- and MRI data as well as the incomplete response with respect to reducing the frequency of myelin peptide-specific T cells in all of the small group of patients with highly active disease indicate that further dose escalation should be explored and that a better understanding of the mechanism/s of action in humans needs to be gained, and it is likely that this will be achieved in the phase IIa trial with a more homogeneous group of patients, who will all be treated with the same dose of cells. Despite these caveats, we believe that tolerization by ETIMS has distinct advantages over other approaches, which include the possibility for blocking epitope spreading and that only a single or few treatment courses are needed. Given the fact, that experimental studies demonstrated efficacy and safety of this approach in different T cell driven autoimmune diseases with defined antigens, allergy and transplantation, antigen-coupled cell tolerance could in principle be applied in several immunopathological conditions in humans,



however further data are clearly needed and it is open at this point if the effects of this treatment are equally broad in humans.

## **Patients and Methods**

### ***Ethics Statement***

The protocol was reviewed and approved by the Ethics Committee of the Hamburg Chamber of Physicians. An independent data and safety monitoring board (DSMB) oversaw the study. All procedures were done following the rules of the Declaration of Helsinki Guidelines and all regulatory steps were performed under guidance of the German regulatory authority for biologics/cell therapies, the Paul-Ehrlich Institute, Langen, Germany. The trial is registered under the EudraCT number 2008-004408-29. All patients signed a written informed consent before inclusion in the study.

### ***Preparation of antigen coupled cells***

Peripheral blood mononuclear cells (PBMC;  $4-10 \times 10^9$ ) and 200 ml autologous plasma were isolated from MS patients by leukapheresis (Cobe Spectra; TerumoBCT, Belgium). Immediately after collection of cells, the manufacturing process was under GMP conditions in the clean room at the Institute of Transfusion Medicine, Center for Diagnostics, University Medical Center Hamburg-Eppendorf, Germany. Briefly, red blood cells were lysed by 15 min incubation in 200 ml ACK lysis buffer (consisting of ammonium-chloride, PhEur [Merck, Darmstadt, Germany], potassium hydrogen carbonate, PhEur [Merck] and water for injection PhEur [Baxter, Germany]) at room temperature. Subsequently, PBMC were washed twice in 200 ml of saline (Baxter)

containing citrate-phosphate-dextrose buffer (CPD, Fresenius, Bad Homburg, Germany). After cell counting,  $1.5-4 \times 10^9$  PBMC were re-suspended in 10-20 ml of saline and 1 ml containing 0.5 mg of each GMP peptide was added (MBP13-32, MBP83-99, MBP111-129, MBP146-170, MOG1-20, MOG35-55, PLP139-154; Bachem AG, Bubendorf, Switzerland, the final concentration of each peptide in the coupling reaction was 0.05 mg/ml). The coupling reaction was initiated by the addition of 100-200 mg freshly prepared water-soluble EDC (AppliChem, Darmstadt, Germany). Following 1h shaking incubation at 4°C, the cells were washed 2 times with 100 ml CPD-saline and re-suspended in 100 ml of autologous plasma for injection.

Coupling of the peptides on the surface of PBMC was verified during validation of manufacturing process and at regular intervals during the trial. Briefly, PLP139-154 was replaced by a biotinylated PLP139-154 peptide. At the end of the manufacturing process coupling of biotinPLP139-154 was visualized by FACS analysis using streptavidin conjugated with APC. The respective drug product was not used for patient injection, because the use of biotin-conjugated peptides is not licensed for use in humans. The binding efficiency was not tested individually for all peptides used in the trial, however from pre-clinical experience as well as the chemical properties of the peptides, it is expected that the coupling is efficient for all peptides.

### ***Trial Design and outcome measures***

Ten patients with RRMS (n=8) or SPMS (n=2) were included in this single-centre, open-label, phase I trial. Patient 9 withdrew from the study at month -2 prior to the treatment for personal reasons. The clinical and demographic data of patients treated with ETIMS cell product is shown in Table 1. Inclusion criteria included the following: ages between 18 and 55 years, RR-MS or

SP-MS disease course, disability score (expanded disability status scale, EDSS) between 1 and 5.5 (38), patients had to be able to provide written informed consent prior to any testing under the protocol and patients had to have a specific T cell response against at least one of the peptides used in the trial (see antigen-specific T cell responses section in patients and methods). All patients included in the trial fulfilled this criterion by showing a T cell response against at least one of the peptides used in the trial. Exclusion criteria included the following: primary progressive MS, pregnancy and breast-feeding, history or actual signs of immunodeficiency, concurrent clinically significant cardiac, immunological, pulmonary, neurological, renal or other major disease, splenectomy and cognitive impairment.

The primary outcome of the study was safety and tolerability and was determined by the (i) number and severity of adverse events (AEs), including (ii) significant changes in vital signs, (iii) changes in blood chemistry and differential blood counts and (iv) aggravation of the disease at month +3.

The first cohort of six patients was followed for one month before study-drug-administration with two consecutive clinical magnetic resonance imaging (MRI) and general physical examinations as well as clinical and laboratory assessments (Fig. 1A). A careful dose escalation was performed in these patients to ensure safety and reduce the risk for individuals (for details on the rationale for the lowest and highest dose see supplementary methods). Each patient received a single infusion of antigen-coupled cells. The first patient received  $1 \times 10^3$ , the second patient  $1 \times 10^5$ , the third patient  $1 \times 10^7$ , the fourth patient  $1 \times 10^8$ , the fifth patient  $5 \times 10^8$  and the sixth  $1 \times 10^9$  antigen-coupled cells. The last three patients (patient 7, 8 and 10) were included in the trial as an amendment to increase dose-escalation, and since safety was already documented more active disease patients were included. Patient 7 received  $1 \times 10^9$  antigen-coupled cells,

patient 8  $2.5 \times 10^9$  and patient 9  $3 \times 10^9$ . In these three patients the observation period before study-drug-administration was extended to 3 months (Fig. 1A). At the day of study-drug-administration, all patients were monitored in a phase I inpatient unit for 24 h (Fig. 1B). The trial design stipulated measurement of the primary outcome at month 3 and additional safety follow-up until month 6 with consecutive clinical, MRI and general physical examinations as well as clinical and laboratory analyses to assess AEs and monitor MS disease activity (Fig. 1A). Hematological analyses including differential blood counts and clinical chemistry were performed in the Institute of Clinical Chemistry, University Medical Center Hamburg-Eppendorf. MS disease course was evaluated by neurological examination and MRI assessing presence of new T2 lesions or new contrast enhancing (CEL) on MRI, occurrence of exacerbations and disability progression. Neurologic function was scored with EDSS (38), the Scripps Neurologic Rating Scale (SNRS) (39) and the Multiple Sclerosis Functional Composite (MSFC)(40). The Multiple Sclerosis Impact Scale (MSIS29) was used as a patient based outcome measures of disability (41).

### ***Magnetic resonance imaging***

The MRI examinations were performed on a 1.5 Tesla MRI scanner MAGNETOM Sonata (Siemens, Germany) with a standard head coil at the Department of Neuroradiology, University Medical Center Hamburg-Eppendorf. The following sequences were obtained: T1 pre- and post gadolinium (0.1mM/kg, Gd-BOPTA); PD/T2-weighted images; FLAIR (fluid attenuated inversion recovery), and diffusion-weighted imaging sequences. All brain MRIs were analyzed by experienced neuroradiologists blinded for the clinical findings.

### ***Flow Cytometric analysis***

Peripheral blood, before and after treatment with ETIMS cell product, was collected in EDTA-tubes. Frequency of different cell subsets was analyzed in whole blood by flow cytometry using different antibody panels. Immune cell subsets (granulocytes, eosinophils, monocytes, B, T, NK and NKT cells) were assessed with anti-CD45 (PE-Cy7, eBioscience, San Diego, CA), anti-CD16 (APC-Cy7, BioLegend, San Diego, CA), anti-CD19 (FITC, BD, Franklin Lakes, NJ), anti-CD14 (V450, BD), anti-CD3 (PerCP, BD) and anti-CD56 (PE, eBioscience). Gating strategy is shown in supplemental Fig.S2. T cell subsets including CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup> Treg (CD4<sup>+</sup> FoxP3<sup>+</sup>), „regulatory“ CD8<sup>+</sup> (CD8<sup>+</sup> CD57<sup>+</sup> ILT2<sup>+</sup>) and „proinflammatory“ CD8<sup>+</sup> (CD8<sup>+</sup> CD161<sup>high</sup>) T cells were analyzed using anti-CD3 (PE-Cy7, eBioscience), anti-CD4 (APC, eBioscience), anti-CD8 (PB, Dako- BIOZOL, Eching, Germany), anti-FoxP3 (PE, Miltenyi), anti-CD25 (APC, eBiosciences), anti-CD57 (FITC, BD), anti-ILT2 (PE, Beckman) and anti-CD161 (APC, Miltenyi) antibodies. The corresponding isotype controls were included in all stainings. Cells were analyzed using a LSR-II flow cytometer (Becton Dickinson, BD) and FACS Diva Software (BD).

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (PAA, Pasching, Austria) and functional phenotype of T cells was evaluated by intracellular cytokine staining as follows, 5x10<sup>5</sup> freshly isolated PBMC were incubated over night in 200 µl x-Vivo15 (Lonza) in a sterile FACS tube. Next day cells were stimulated with PMA (50 ng/ml, Sigma, St Louis, USA) and ionomycin (1 µg/ml, Sigma) in the presence of brefeldin A (10 µg/ml, eBioscience) for 5 h. After washing with PBS cells were stained with LiveDead kit (AmCyan, Invitrogen), fixed, permeabilized and stained with different antibodies: anti-IL-17 (Alexa 647; ebiosciences), -IL-4 (PE-Cy7, Biolegend), -IFN-γ (FITC, Biolegend), -

IL-10 (PE; Biolegend), -CD3 (PE, DakoCytomation), -CD4 (PB, DakoCytomation) and -CD8 (PB, Biolegend) or with the corresponding isotype controls.

### ***Antigen-specific T cell responses***

The antigen-specific T cell responses towards the myelin peptides used in the study were measured in freshly isolated PBMC prior to the tolerization procedure and after 3 months. Antigen-specific T cell responses were analyzed by proliferation assays using thymidine incorporation. Briefly, isolated PBMC were seeded in 96 wells plates at  $1.5 \times 10^5$  PBMC/well in x-Vivo-15 (Lonza, Basel, Switzerland) medium with  $1 \mu\text{M}$  of each peptide. 48 wells were seeded per antigen and 6 wells only with medium as negative control in each plate. Tetanus toxoid (TTx,  $5 \mu\text{g/ml}$ ) (Novartis Behring, Marburg, Germany) was used as positive control. On day 7, plates were incubated for 15 h with  $1 \mu\text{Ci}$  of  $^3\text{H}$ -thymidine (Hartmann Analytic, Braunschweig, Germany).  $^3\text{H}$ -Thymidine pulsed plates were analyzed with a scintillation beta counter (Wallac 1450, PerkinElmer, Rodgau-Jürgesheim, Germany). The scintillation counts (CPM) of each well were measured. Wells showing CPM higher than the mean plus 3 standard deviations ( $\text{mean} + 3 \times \text{STDEV}$ ) of the unstimulated wells were considered as positive.

### ***Statistical analysis***

Statistical analysis was done using GraphPad Prism 4 (GraphPad Software Inc.) software. Descriptive statistics are reported as mean  $\pm$  SEM. The comparisons of clinical and immunological parameters were performed for two-group comparisons using a paired t-test. Comparisons of three groups and more were assessed by one-way ANOVA with Bonferroni's

correction for multiple comparisons or Kruskal-Wallis test with Dunn's post test depending on the distribution of the data. P-values <0.05 were considered statistically significant.

### ***Supplementary materials***

Fig S1: Schematic representation of the manufacture process.

Fig S2: Gating strategy for blood cell subsets.

Fig S3: CD4 and CD8 subsets in high-dose patients before and after treatment.

Supplementary Methods: Rationale for choosing the lowest and highest dose.

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**Acknowledgements:** We thank H. McFarland (Scientist Emeritus, Neuroimmunology Branch, NINDS, NIH, Bethesda, USA), R. Gold (Department of Neurology, Ruhr-University Bochum, Germany) and N. Kroeger (Center for Stem Cell Transplantation, University Medical Center Hamburg Eppendorf, Germany) for their participation as members of the DSMB. We thank M. Daumer (Sylvia Lawry Center for MS Research, Munich, Germany) for data management and T. Eiermann and T. Binder (Institute of Transfusion Medicine, Department of Clinical Theoretical Medicine, University Medical Center Hamburg-Eppendorf, Germany) for HLA genotyping.

**Funding:** A. Lutterotti was supported by a fellowship of the Alexander von Humboldt Foundation. inims is supported by the Gemeinnützige Hertie Foundation. The ETIMS project was largely supported by a grant of the German Federal Ministry for Education and Research (BMBF), and received additional support from the Cumming Foundation, Salt Lake City, Utah, USA. SD Miller received travel support from the Myelin Repair Foundation. **Author**

**contributions:** Study concept and design: AL, SDM, MS, RM; Patient management: JPS, CH, SS. Manufacture process development: AL, AS, KHS, SR. Mechanistic studies: SY, KHS, PB, SR, CS, MS. MRI analysis: MB. Drafting of the manuscript: AL, MS, RM. Critical revision of the manuscript: AL, SY, AS, JPS, CH, SS, SDM, MS, RM. Study supervision: AL, KHS, JPS, SDM, MS, RM. **Competing interests:** A Lutterotti, SD Miller and R Martin are listed as co-inventors on a University Medical Center Hamburg-Eppendorf patent related to the use of antigen-coupled cells in MS.

## **Figures:**

**Fig. 1. Study Design.** A) The first 6 patients were followed before treatment with two clinical-

MRI- and laboratory examinations at month -1 before treatment and day 0 and after treatment at weeks 2, 6 and 3 months. Vital signs and AEs were recorded during infusion, 30 min, 1, 2, 4 h after infusion, on day +1, day +3, week +2, week +3, week +6 and month +3. General physical examination was performed at month -1 and day +1. Neurological examination was done at month -1, day -1, week +2, week +6, and month +3. Brain MRI was assessed at month -1, day -1, week +2, week +6 and month +3. MSIS29 was assessed at month -1, day +1, week +6 and month +3. Patients were further followed for AEs, serious AEs (SAE), clinical and MRI disease activity at months +4, +5 and +6. The last three patients were followed at four time-points (month -3 to D0) before treatment and further examinations were performed at week 2, months 1, 2, 3 and 6 (scattered line). B) Patients were admitted to the Phase I unit the day prior to treatment. At D0 leukapheresis was performed and the autologous cell product manufactured under GMP conditions. Following quality control study drug was infused the same day and patients monitored for AEs for 24 hours. C) Panel depicts the most important steps of the manufacturing process, which was performed in blood bags while maintaining a closed system. Erythrocytes are lysed with lysis buffer. The peptides are added and the coupling procedure is initiated by addition of EDC. Finally quality control is performed. As soon as all release criteria are fulfilled, the ETIMS cell product is used for therapy. \* includes washing steps and cell counting. QC: quality control.

**Fig. 2. Peripheral blood cell counts after ETIMS treatment.** Absolute peripheral blood counts (mean  $\pm$  SEM) of eosinophils, basophils, neutrophils, lymphocytes, monocytes and platelets before treatment (month -1 and day -1) and after treatment (day 1, week 2, week 6 and month 3). Dotted line indicates the time point of study-drug-administration.

**Fig. 3. Clinical exacerbations after ETIMS treatment.** Clinical exacerbations in the year before treatment (grey circles), during the first three months after treatment (black circles) and during the safety follow-up until month 6 for the nine patients included in the trial. Dose of antigen-coupled cells is shown on the right side. Dotted line indicates the time point of study drug administration.

**Fig. 4. New T2 lesions and CEL after ETIMS treatment.** New T2 lesions (left graphs) and CEL (right graphs) in the first cohort of patients with low disease activity (patients 1-6) (upper graphs), and in the last three patients with higher disease activity (patient 7, 8 and 10) (lower graphs). Mean  $\pm$  SEM is shown in the two upper graphs corresponding to patients 1-6. T2 lesions graphs: y-axis is number of T2 lesions, first column in grey indicates the number of total T2 lesions three months before study-drug-administration, columns in black indicate new T2 lesions before treatment (months -2, -1 and 0) and after treatment (week 2, months 2, 3, 4, 5 and 6). CEL graphs: y-axis is number of new CEL lesions (both brain and spinal cord CEL), new CEL before treatment (month -3, -2, -1 and 0) and after treatment (week 2, month 2, 3, 4, 5, and 6) are shown. Dotted line indicates the time point of study drug administration.

**Fig. 5. Neurologic function after ETIMS treatment.** EDSS (left graphs), SNRS (middle graphs) and MSIS29 (right graphs) in the first cohort of patients with low disease activity (patients 1-6) (upper graphs), and in the last three patients with higher disease activity (patient 7, 8 and 10) (lower graphs) before treatment (months -3 and -1) and after treatment (week 2,

months 3 and 6). Mean  $\pm$  SEM is shown in the three upper graphs corresponding to patients 1-6. Dotted line indicates the time point of study drug administration.

**Fig. 6. Immune cell subsets after ETIMS treatment.** Percentage of B (CD45<sup>+</sup> CD19<sup>+</sup>), monocytes (CD45<sup>+</sup> CD14<sup>+</sup>), T (CD45<sup>+</sup> CD3<sup>+</sup> CD56<sup>-</sup>), NK (CD45<sup>+</sup> CD3<sup>-</sup> CD56<sup>+</sup>) NKT (CD45<sup>+</sup> CD3<sup>+</sup> CD56<sup>+</sup>) cells (**A.**), CD4<sup>+</sup> T cells (CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup>) (**B.**) and CD8<sup>+</sup> T cells (CD3<sup>+</sup> CD4<sup>-</sup> CD8<sup>+</sup>) (**C.**) measured by flow cytometry in the four patients receiving the higher dose of antigen couple cells (patients 6, 7, 8 and 10) before (month -1 and day -1) and after (week 2 and month 3) ETIMS treatment. Percentage of Th1 (CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup>), Th17 (CD4<sup>+</sup> IL-17<sup>+</sup>), Th2 (CD4<sup>+</sup> IL-4<sup>+</sup>), Treg (CD4<sup>+</sup> FoxP3<sup>+</sup>) and Tr1 (CD4<sup>+</sup> IL-10<sup>+</sup>) (**B.**) and “regulatory” CD8<sup>+</sup> (CD8<sup>+</sup> CD57<sup>+</sup> ILT2<sup>+</sup>) and “proinflammatory” CD8<sup>+</sup> (CD8<sup>+</sup> CD161<sup>high</sup>) (**C.**) T cells measured by flow cytometry in the four patients receiving the higher dose of antigen-coupled cells (patients 6, 7, 8 and 10) before (month -1) and after (month 3) ETIMS treatment. Mean  $\pm$  SEM is shown. Dotted line indicates the time point of study drug administration.

**Fig. 7. Myelin specific T cell response after ETIMS treatment.** **A)** MOG 1-20, MOG35-55, MBP 13-32, MBP 83-99, MBP 111-129, MBP 146-170, PLP 139-154, TTx -specific T cell responses and unstimulated wells before (month -1) and after (month 3) ETIMS treatment in patients treated with low ( $1 \times 10^3 - 5 \times 10^8$ , patients 1-5; left panel) or high ( $1 \times 10^9 - 3 \times 10^9$ , patients 6, 7, 8 and 10; right panel) dose of antigen-coupled cells. Proliferative responses were measured by <sup>3</sup>H-thymidine incorporation assay. Graphs (y-axis) represent the scintillation counts per minute (CPM). The dotted line represents the threshold set for the mean + 3x standard of unstimulated wells. All wells above this threshold are shown in red.



## Supplementary Materials:

**Fig. S1. Schematic representation of the manufacture process.** Autologous PBMC are collected by leukapheresis and subsequently pulsed with 7 myelin peptides in the presence of EDC. The whole procedure including the washing steps is performed in standard blood bags. After quality control the autologous cell product is infused the same day.

**Fig. S2. Gating strategy for blood cell subsets.**  $CD45^+$  cells (leukocytes),  $CD16^{high}$  (granulocytes),  $CD45^+ CD16^-$  (leukocytes without granulocytes),  $CD45^+ CD16^- CD19^+$  (B cells),  $CD45^+ CD16^- CD14^+$  (monocytes),  $CD45^+ CD16^- CD14^- CD19^- CD3^+ CD56^-$  (T cells),  $CD45^+ CD16^- CD14^- CD19^- CD3^+ CD56^+$  (NKT cells) and  $CD45^+ CD16^- CD14^- CD19^- CD3^- CD56^+$  (NK cells).

## Fig. S3. $CD4^+$ and $CD8^+$ T cell subsets after ETIMS treatment

Percentage of  $CD4^+$  T cell subsets (left panel) Th1 ( $CD4^+ IFN-\gamma^+$ ), Th17 ( $CD4^+ IL-17^+$ ), Th2 ( $CD4^+ IL-4^+$ ), Treg ( $CD4^+ FoxP3^+$ ) and Tr1 ( $CD4^+ IL-10^+$ ) and  $CD8^+$  T cell subsets (right panel) “regulatory”  $CD8^+$  ( $CD8^+ CD57^+ ILT2^+$ ) and “proinflammatory”  $CD8^+$  ( $CD8^+ CD161^{high}$ ) T cells measured by flow cytometry in the four patients receiving the higher dose of antigen-coupled cells (patients 6, 7, 8 and 10) before (month -1) and after (month 3) ETIMS treatment. Mean  $\pm$  SEM is shown.

**Supplementary Methods:** Rationale for choosing the lowest and highest dose.

At the beginning of the developmental process we aimed for a maximum dose of  $1 \times 10^9$  antigen

coupled cells. This target cell number was based on calculations derived from both in-vivo and in-vitro data taking into account both efficacy and safety following the considerations outlined below:

1) In pre-clinical studies in mice, the efficacy of the treatment with antigen-coupled cells has been assessed by comparing antigen-specific recall responses by DTH between syngeneic SJL mice compared to sham-treated mice. Different doses of antigen-coupled cells have been tested ranging from  $5 \times 10^6$  to  $1 \times 10^8$  cells. In SJL mice  $1 \times 10^7$  injected donor cells were the lowest amount of cells with a significant difference in ear swelling compared to controls. Thus we deduced this dose as minimum effective dose in mice. If this minimally effective dose is taken to estimate the minimal anticipated biological effect level (MABEL), the following dose represents the MABEL:  $1 \times 10^7/20\text{g} = 30 \times 10^9/60\text{kg}$ , and after adjustment for the human equivalent dose (HED), i.e. division by 12.3, the final MABEL dose in humans is  $2.44 \times 10^9$  cells/60 kg.

2) The level with no observable adverse effect (NOAEL) was calculated according to the Guidance for Industry and Reviewers, Estimating the Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers. In mice we have not observed any adverse effects injecting  $5 \times 10^7$  cells ( $5 \times 10^7/20\text{g} = 2,5 \times 10^9$  cells/kg), which is the human equivalent dose (HED) based on conversion mg/kg (divide mouse dose by 12.3) of  $12 \times 10^9$  cells per 60 kg person.

3) The in-vitro potency determinations showed a reduction of antigen-induced proliferation, i.e. a pharmacodynamic effect, to approximately 55% at a ratio of 1:8 between antigen-specific T cell clone and EDC-fixed, peptide-pulsed PBMCs (tPBMCs). If we were to base our MABEL estimates on these in-vitro data in humans, we considered the following: Our and other groups' extensive studies on the precursor frequencies of myelin antigen-specific autoreactive T cells in

the peripheral blood of humans have shown the following precursor frequencies: If frequency estimates are based on data generated with ELISPOT (interferon-gamma secreting cells upon stimulation with myelin peptide (Olsson et al., 1990 and 1992) frequencies ranged between 1/10,000 PBMC to 1/100,000 PBMC. Most studies including ours used limiting dilution-based in-vitro culture systems and generated the following frequencies for T cells specific for an individual myelin peptide or protein: 1/100,000 PBMC (as the highest) to between 1/1.000,000 and 1/10.000,000 PBMC (the latter is the range that was most frequently observed, (Martin et al., 1993, Bielekova et al., 2000).

Hence the estimated minimally required number of tPBMCs to tolerize an individual antigen-specific T cell clone in-vivo is 125 cells. Since the tPBMCs to be used under ETIMS are pulsed with 7 peptides, this number probably needs to be multiplied by 7, i.e. 875 tPBMCs. This number is  $11.4 \times 10^4$  lower than the minimally effective in-vivo dose of antigen-coupled cells in mice (i.e.  $1 \times 10^7$  antigen-coupled cells; see above).

4) If we consider both this minimally effective in-vivo dose of antigen-coupled cells in mice (i.e.  $1 \times 10^7$ ; see above) and the above calculated putative minimally effective in-vivo dose of tPBMCs in humans to tolerize individual autoreactive TCCs (875 tPBMCs), we arrive at the following range for MABEL:

mouse-derived number for MABEL:  $2.44 \times 10^9$  tPBMCs/60 kg

human-derived number for MABEL\*: 875 tPBMCs/60 kg

In summary of the above, we considered the following for the first-in-man trial. In the phase I and dose finding, the maximum recommended starting dose would be the one derived from the

mouse-derived number for MABEL ( $2.44 \times 10^9$  tPBMCs/60 kg), however at that time we were afraid that it will be difficult to reach  $2 \times 10^9$  tPBMCs for the trial (and therefore our treatment dose has been set at  $1 \times 10^9$  tPBMCs).

The maximum recommended starting dose from the human-derived number for MABEL is 875 tPBMCs/60 kg. Therefore, we have stipulated the following starting dose and tPBMCs numbers for the dose finding:

First patient:	$0.875 - 1 \times 10^3$ tPBMCs (which is the MABEL derived from the human numbers assuming the lowest number of autoreactive precursor cells from the literature, i.e. $1 \times 10^{-7}$ cells, see above)
Second patient:	$1 \times 10^5$ tPBMCs
Third patient:	$1 \times 10^7$ tPBMCs
Fourth patient:	$1 \times 10^8$ tPBMCs
Fifth patient:	$5 \times 10^8$ tPBMCs
Sixth patient:	$1 \times 10^9$ tPBMCs

After the experience with the first six patients and the favorable safety and tolerability data we chose to expand the dose escalation to reach the mouse-derived MABEL ie.  $2.44 \times 10^9$  tPBMCs. Despite our careful attempts to estimate MABEL and NOAEL from mouse data to humans, it is clear that there remain uncertainties, because validated prior evidence for such equivalence calculations do not exist for cell-based therapies and particularly do not take into account the substantial differences between the immune systems in rodents and

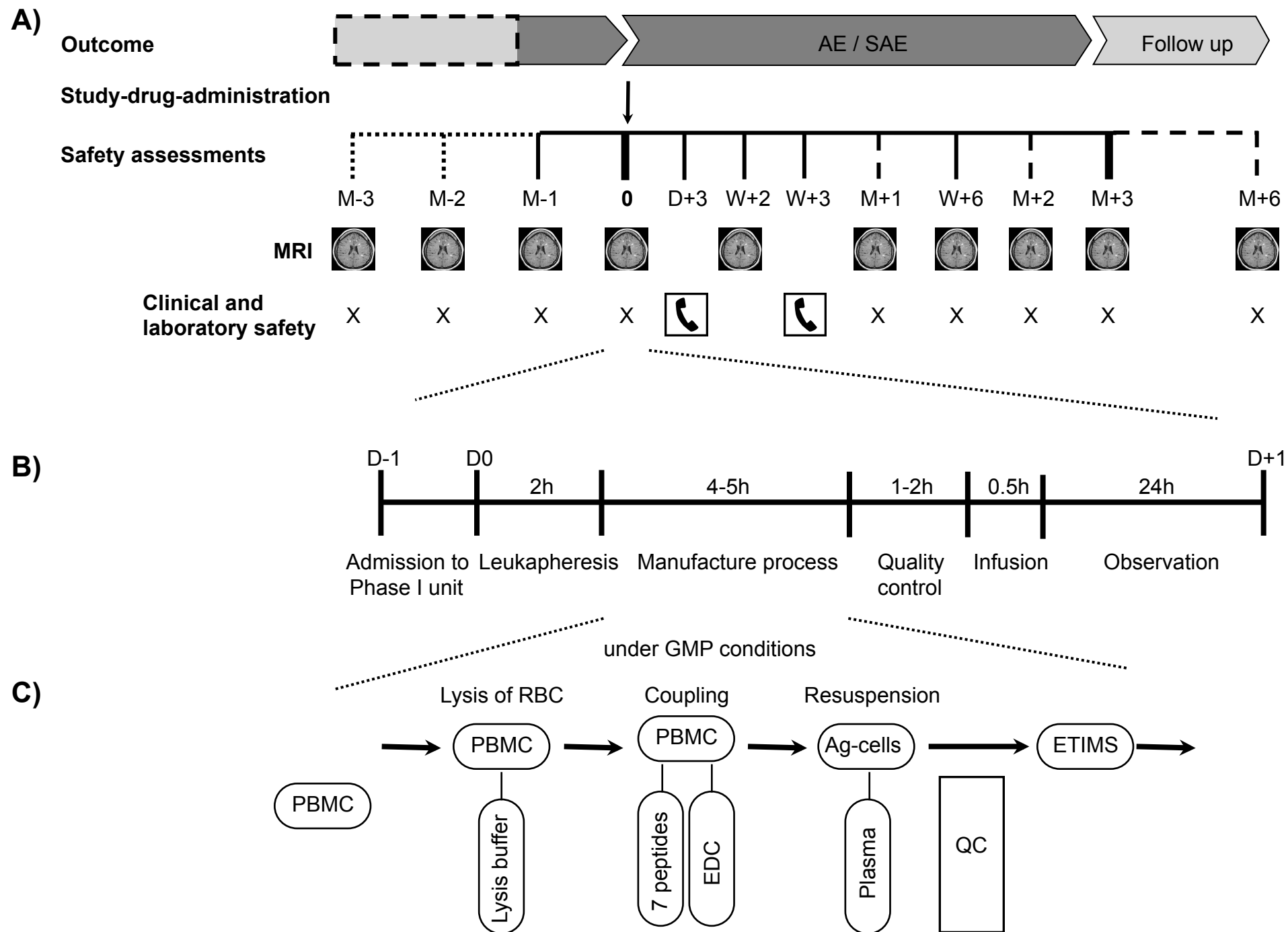


Figure 1

Figure 2

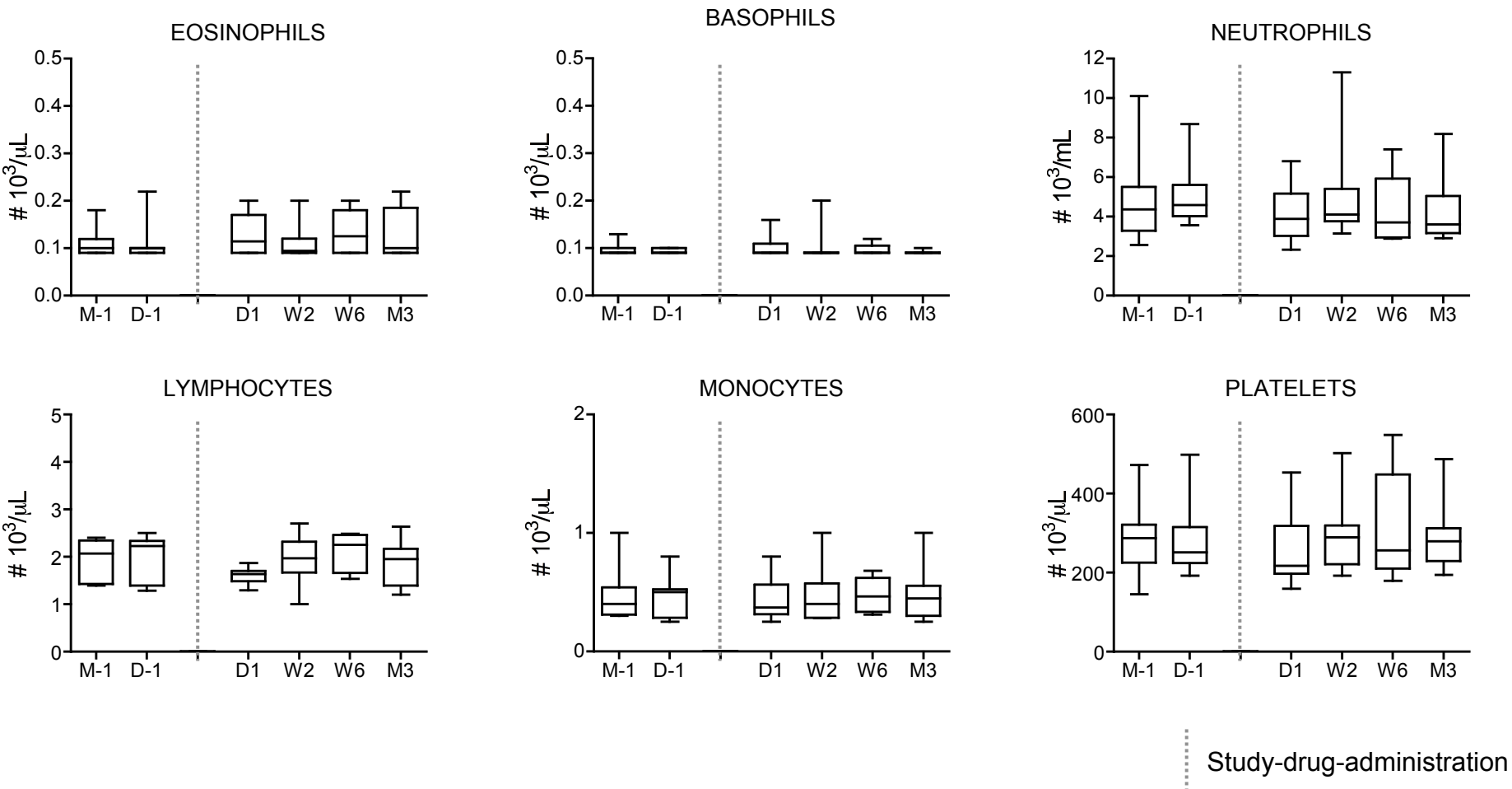


Figure 3

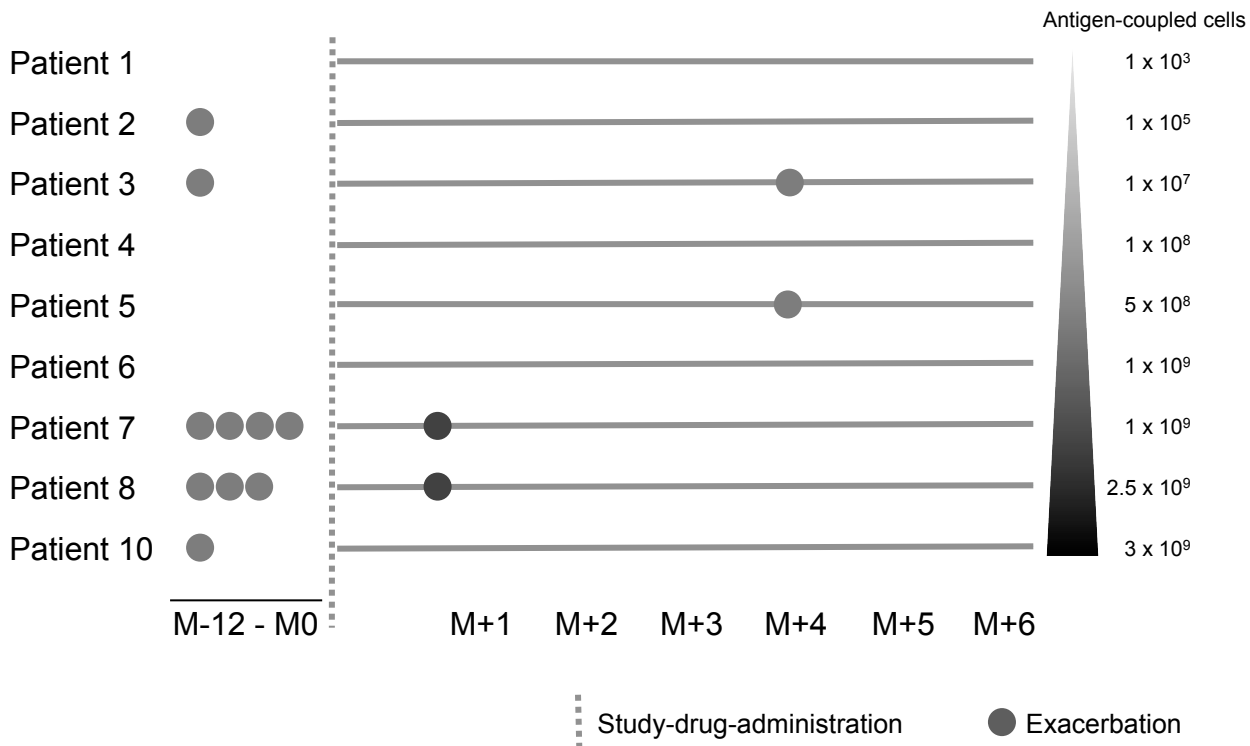
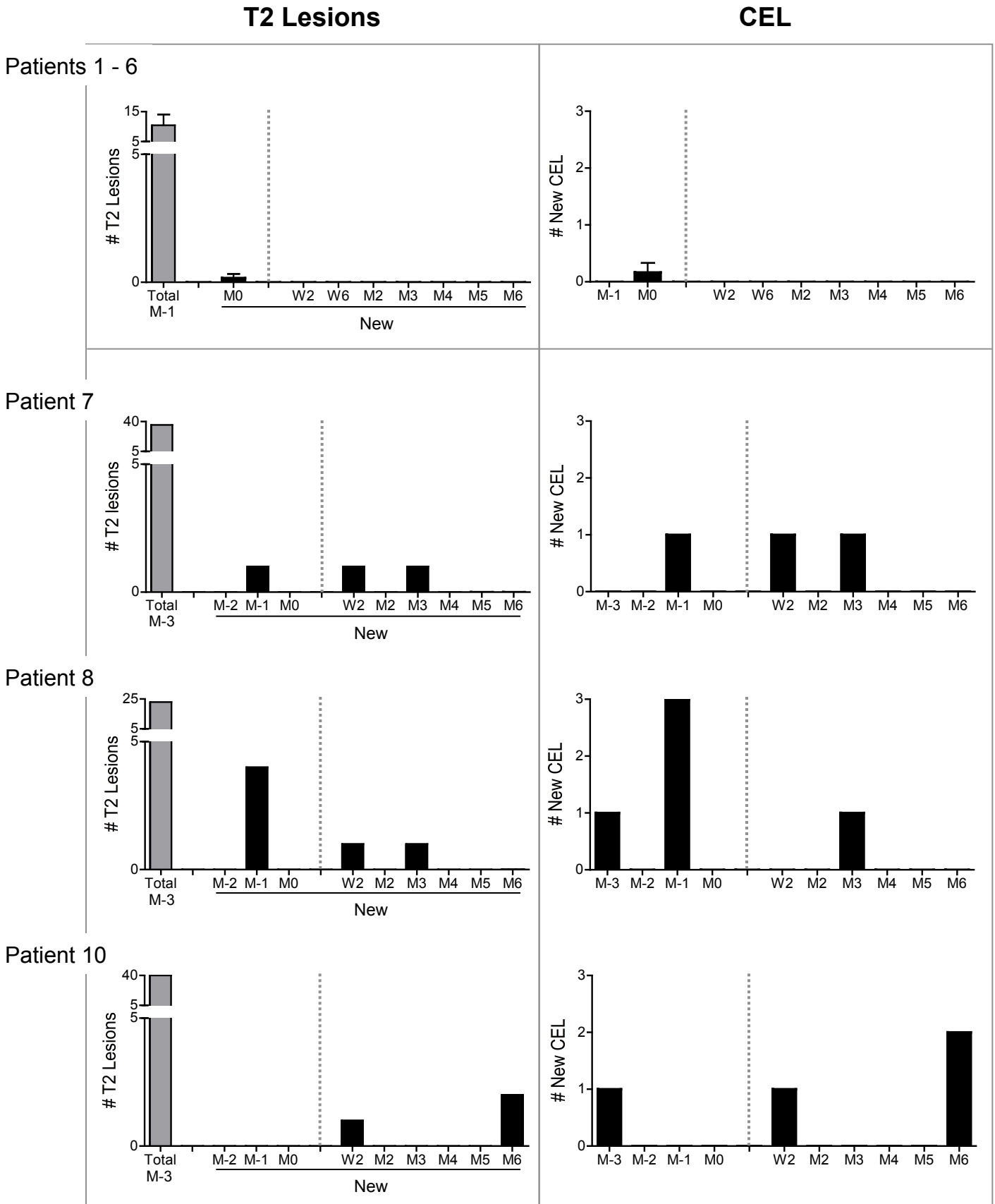


Figure 4

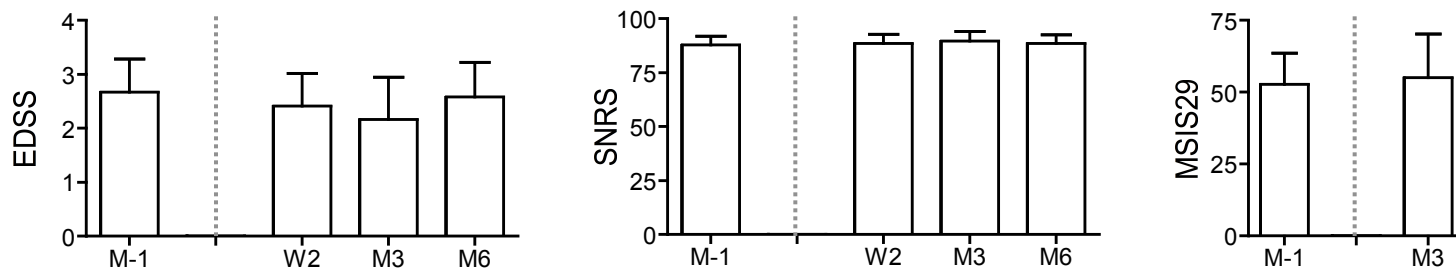


Study-drug-administration

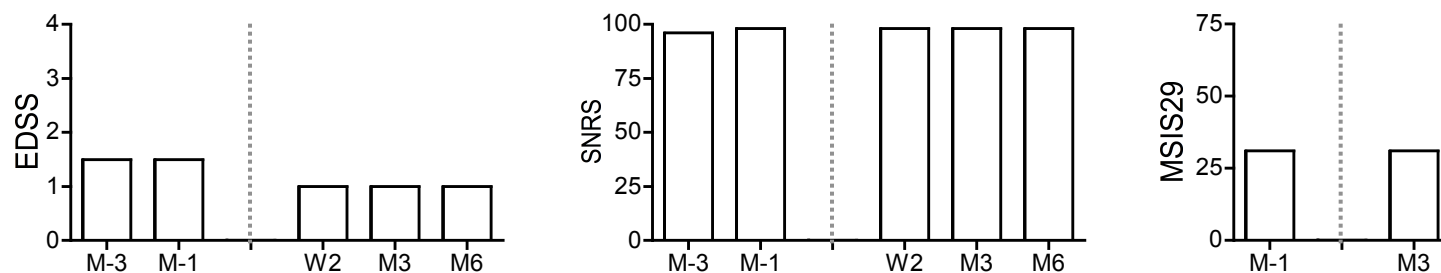


Figure 5

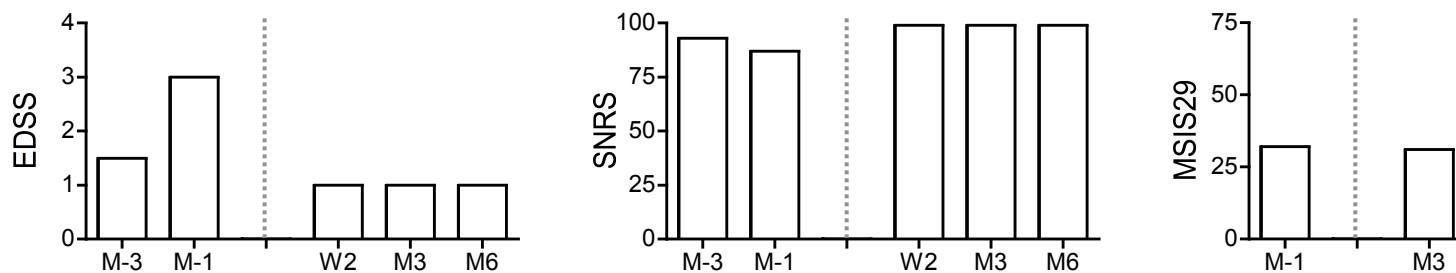
Patient 1 - 6



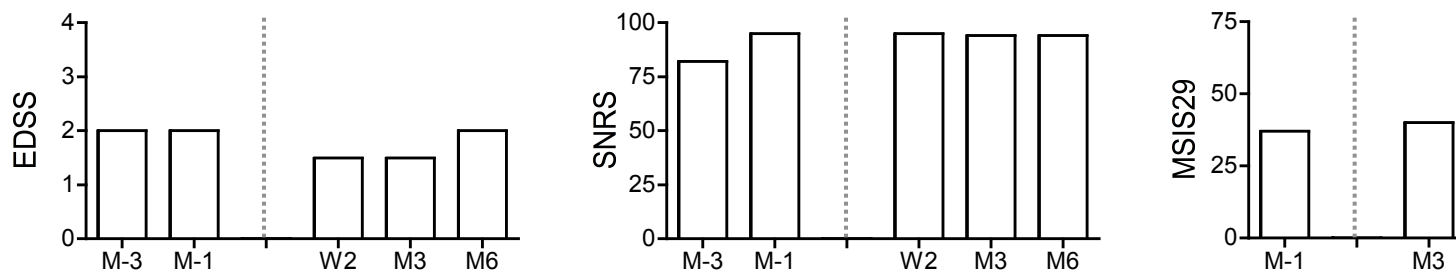
Patient 7



Patient 8



Patient 10



Study-drug-administration

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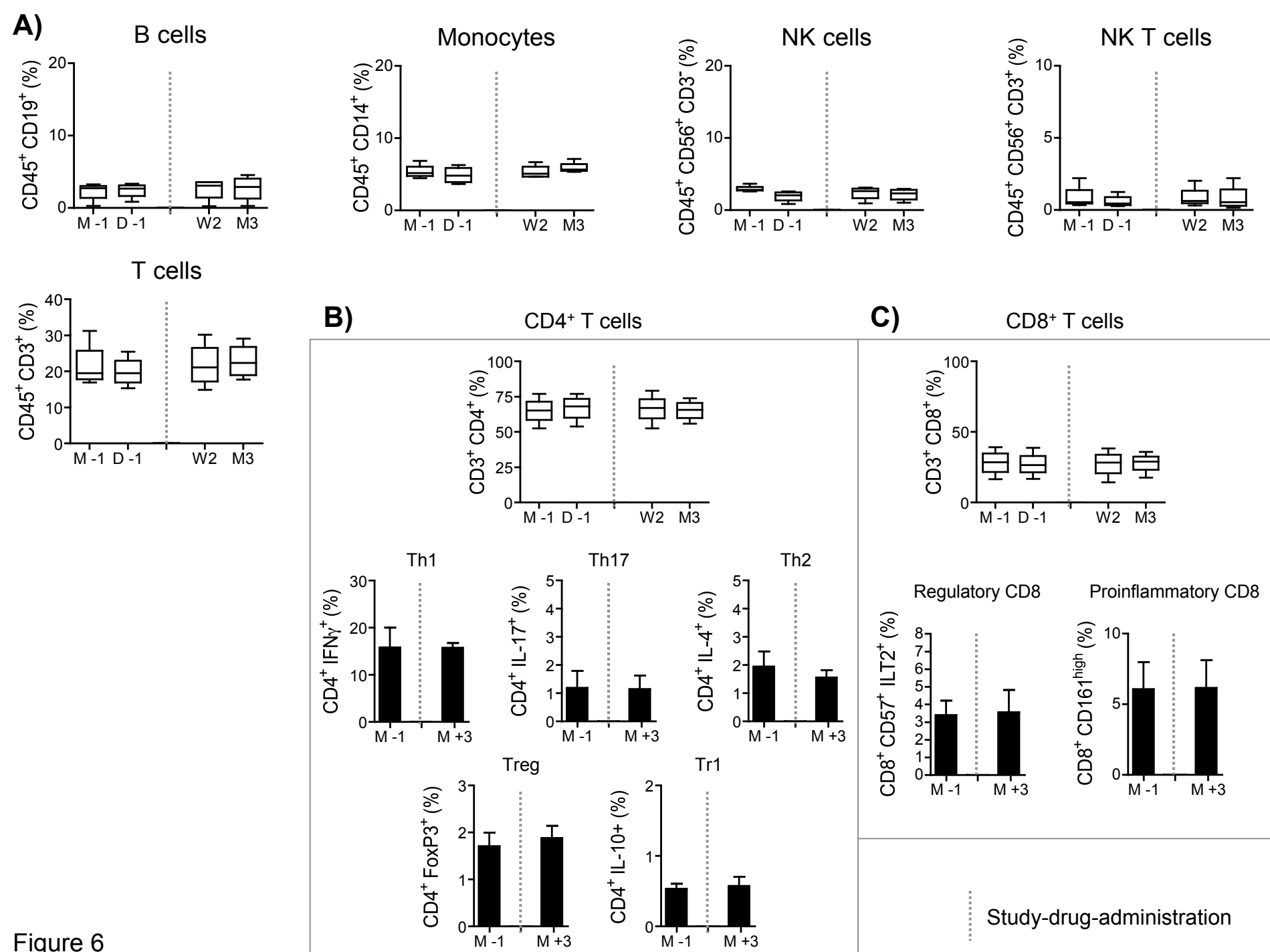


Figure 6

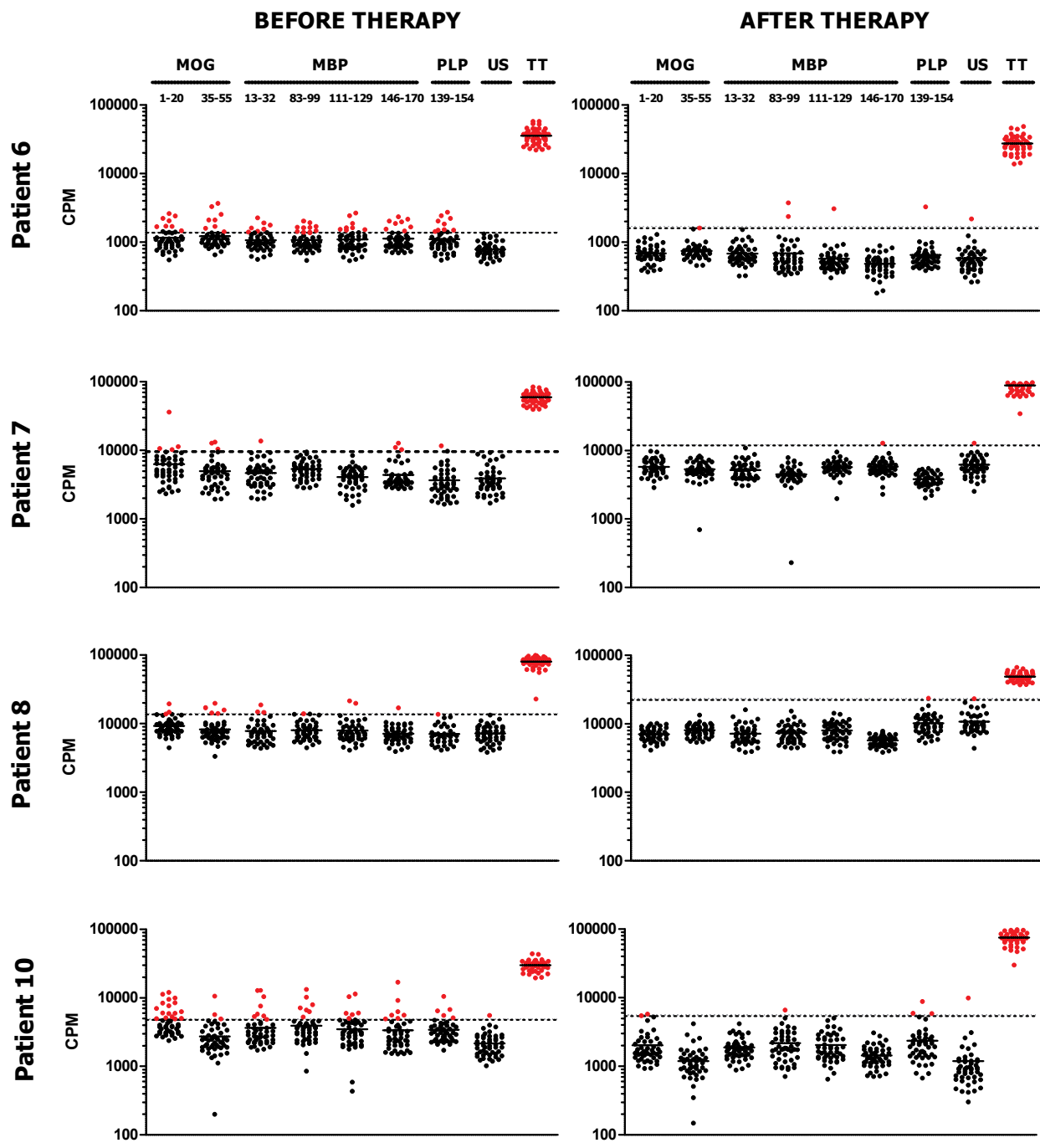
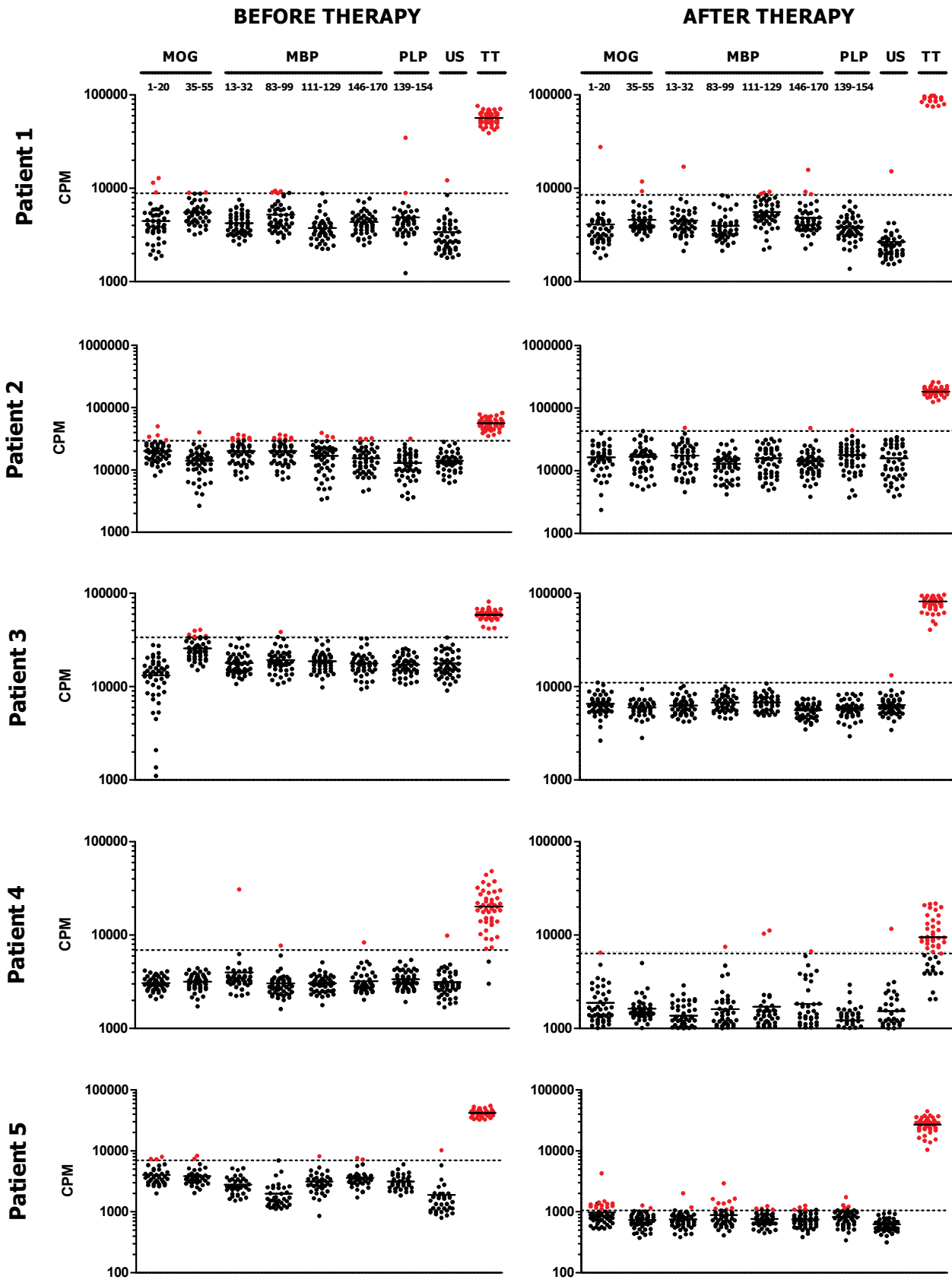
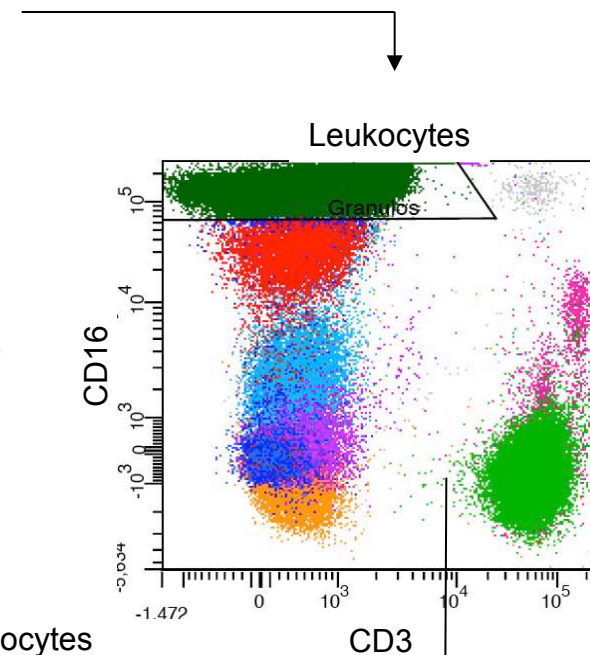
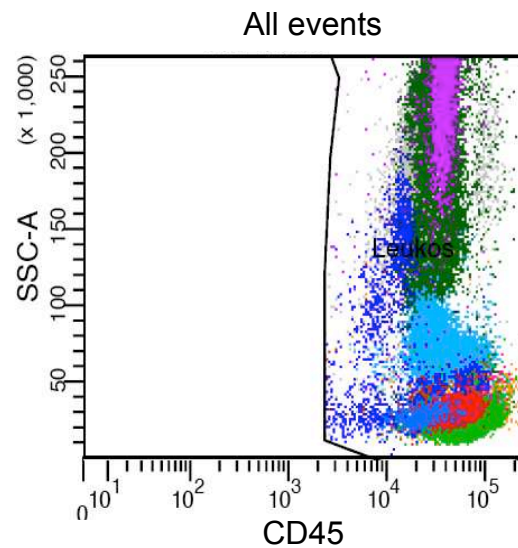
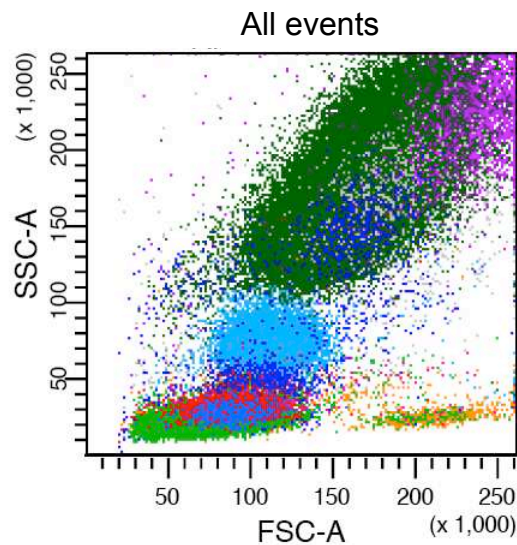
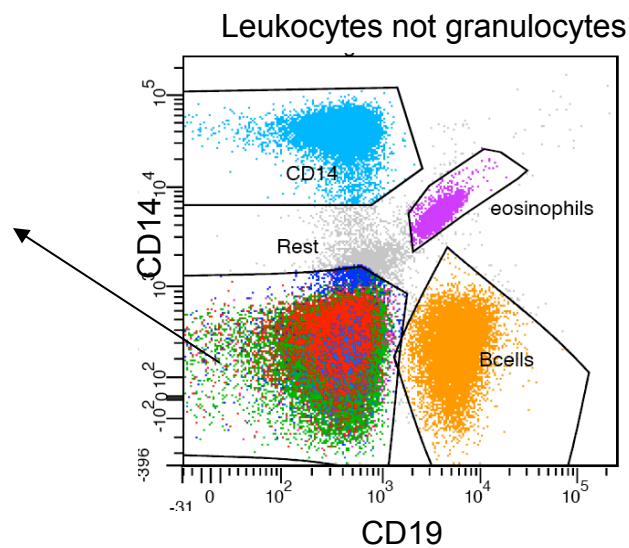
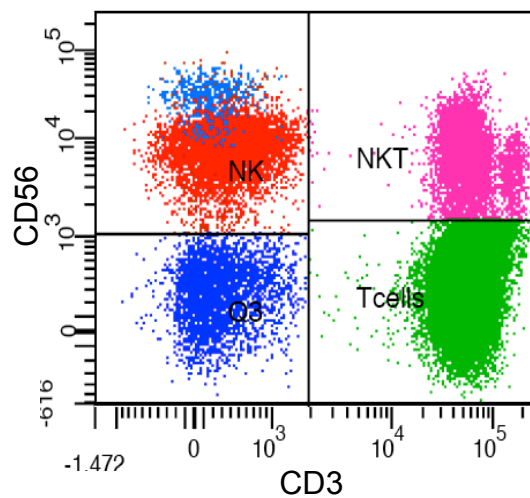


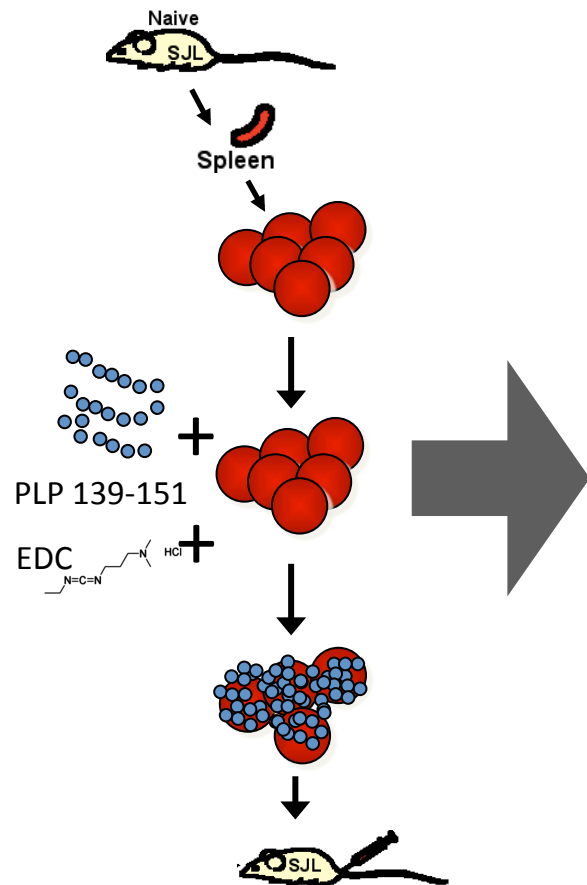
Figure 7



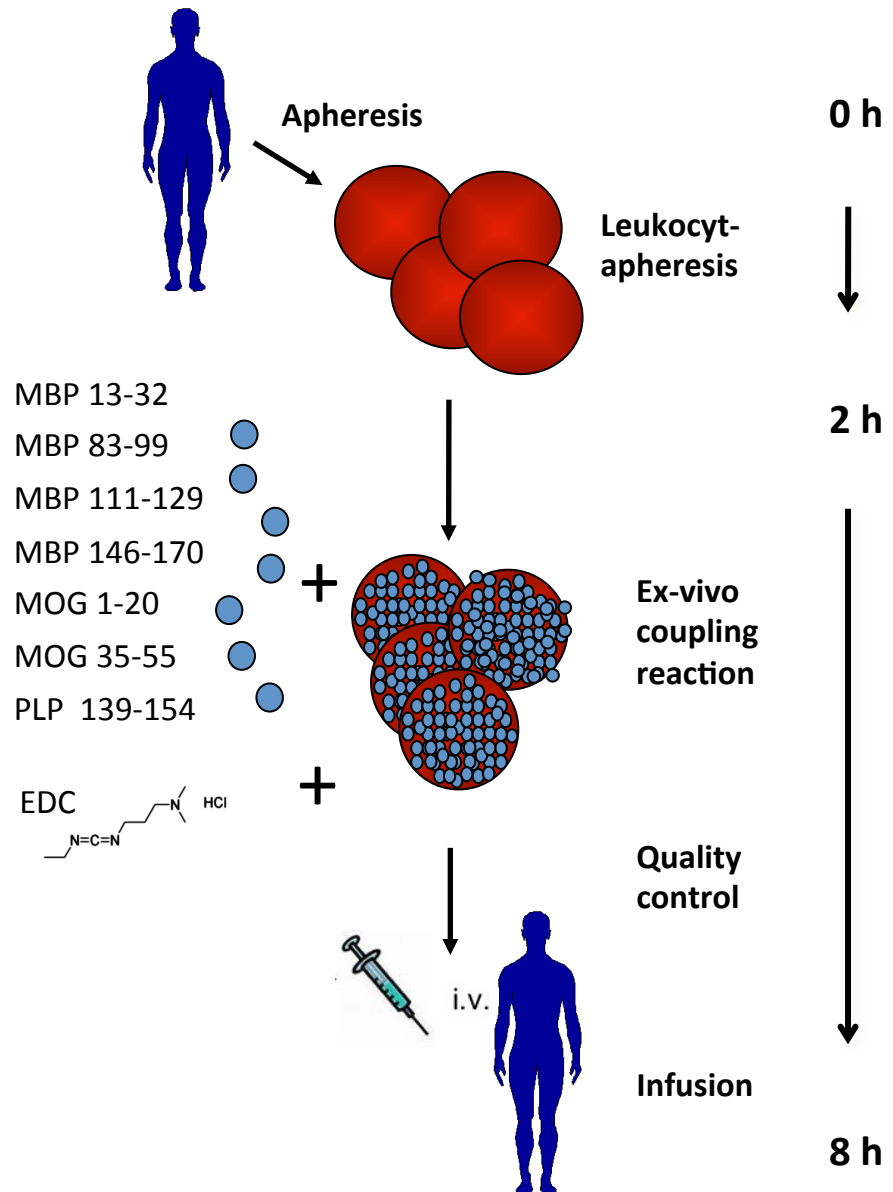
Leukocytes, not granulocytes, not monocytes, not B cells and not eosinophils



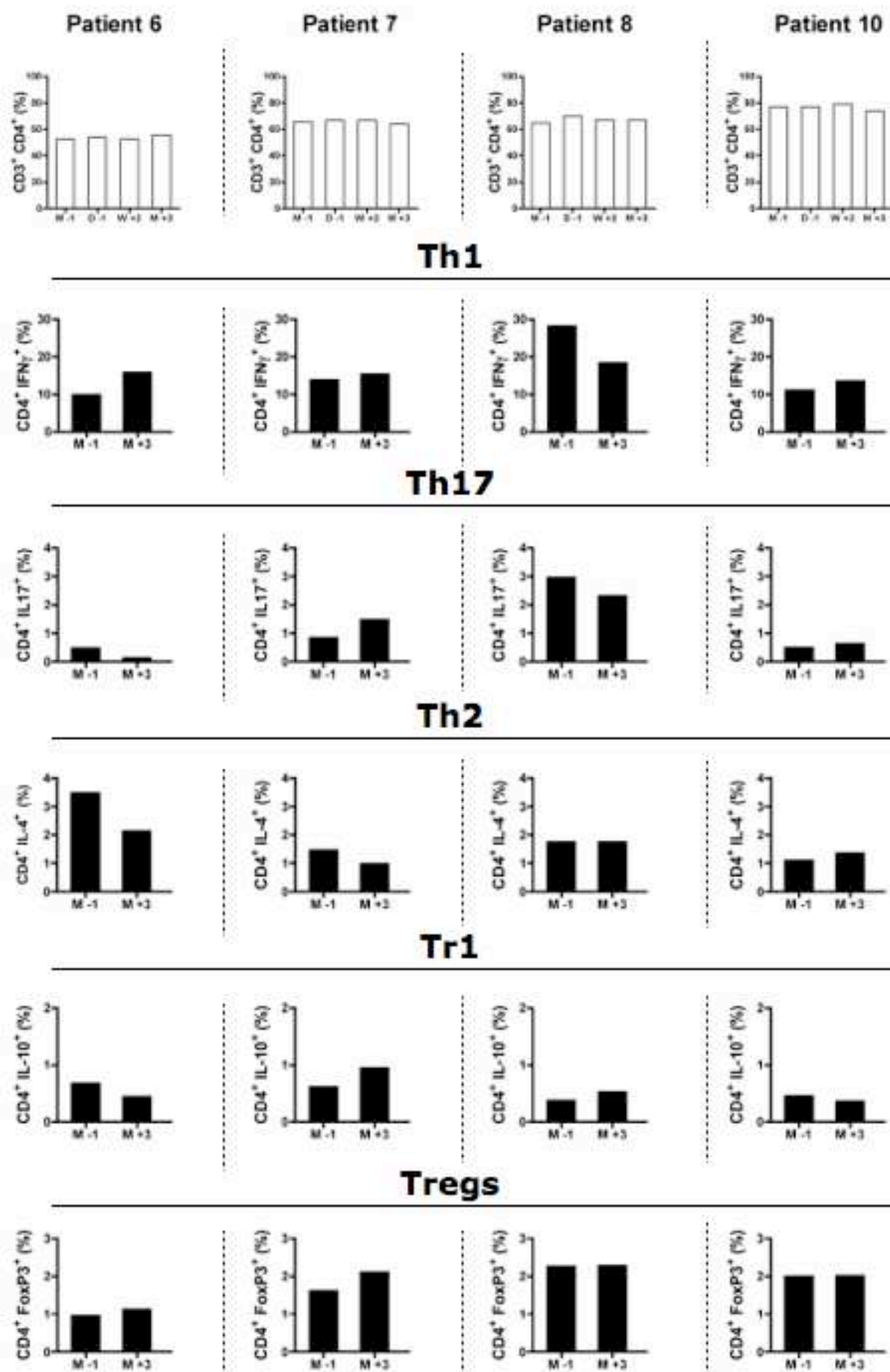
## Use of peptide-pulsed, fixed splenocytes in the EAE model



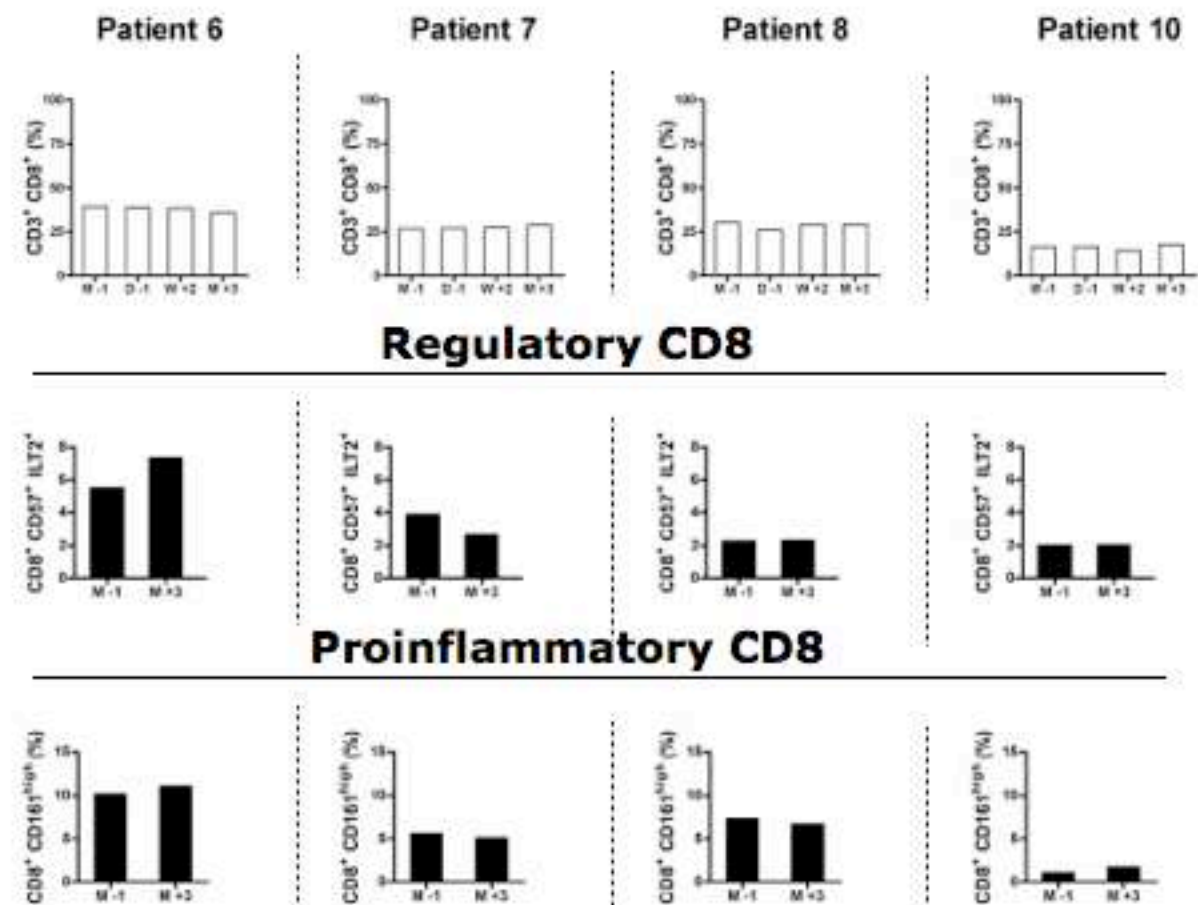
## Use of peptide-pulsed, fixed PBMC in MS patients



## CD4+ T cells



## CD8+ T cells



**Figure S3**